JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1992, p. 418–426
0095-1137/92/020418-09$02.00/0

Vol. 30, No. 2

Comparison of Various Detection Methods for Periodontopathic Bacteria: Can Culture Be Considered the Primary Reference Standard?

WALTER J. LOESCHE,[1]* DENNIS E. LOPATIN,[1] JANICE STOLL,[1] NEAL VAN POPERIN,[1] AND PHILIPPE P. HUJOEL[2]

Department of Biological and Materials Sciences, The University of Michigan School of Dentistry, Ann Arbor, Michigan 48109-1078,[1] and Department of Dental Public Health Sciences, SM-35 School of Dentistry, University of Washington, Seattle, Washington 98115[2]

Received 9 July 1991/Accepted 14 November 1991

The development of diagnostic tests for a periodontal infection raises the issue as to what the appropriate reference standard, or "gold standard," should be for the evaluation of a new test. The present research was initiated to compare the ability of several detection methods, i.e., a serial dilution anaerobic culture and/or microscopic procedure, a DNA probe procedure, and immunological reagents using both an enzyme-linked immunosorbent assay and an indirect immunofluorescence assay to detect *Treponema denticola*, *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Actinobacillus actinomycetemcomitans* in subgingival plaque samples taken from 204 periodontally diseased tooth sites. The prevalence of the four monitored species varied as a function of both the species and the detection method. Spirochetes were present in 99% of the plaques, whereas *A. actinomycetemcomitans* was detected at the lowest frequency. The culture method yielded the lowest prevalence values for the three cultivable species. This raised the question as to which results, those obtained by culture or those obtained by the DNA probes and the immunological reagents, were the most reliable. This issue was addressed by looking at the prevalence profile of the monitored organisms, as determined by all the detection methods. If the species was detected by three or four of the detection methods, then it was considered present, whereas if it was absent by three or four of the detection methods, then it was considered absent. This approach showed the DNA probes and immunological reagents to be significantly superior ($P < 0.05$) to the culture approach for the detection of *P. gingivalis*, *A. actinomycetemcomitans*, and *B. forsythus* and to be comparable to the microscopic approach in the detection of *T. denticola*.

The development of diagnostic tests for either periodontal infection or inflammation raises the issue as to what the appropriate reference or primary standard should be for the evaluation of the new test (4, 19). In the case of a periodontal infection(s), the commonly used anaerobic cultural procedures may be inadequate as a primary standard, as certain of the putative periodontal pathogens are either uncultivable or extremely difficult to cultivate, e.g., spirochetes (28) and *Bacteroides forsythus* (12, 38). In addition, the anaerobic procedures themselves have many sources of method error (5, 24, 25, 34, 37).

The inability to reliably culture these organisms has led to the development of either immunological reagents (2, 12, 30, 32, 42) or DNA probes (7, 9, 29, 35) to detect and quantify these organisms. The detection level (microbiological sensitivity) and absence of cross-reactions with other organisms (microbiological specificity) of these assays are usually determined in vitro by using pure cultures of the sought-after organism. The assays are then evaluated for their ability to detect the target organisms in dental plaque. In some studies the ability of the new procedure to detect the target organisms in plaque is simultaneously compared with that of an established procedure, such as culturing. When such comparisons have been performed, the new procedure usually has a high sensitivity (true positive) and a low specificity (true negative) relative to the culturing procedure (19, 29, 42). The low specificity is due to the large number of positive plaques with the new procedure, compared to the smaller number in the cultural procedure. This gives rise to a high proportion of false-positive results, either meaning that the new procedure is detecting species other than the target organism or that the culturing procedure is deficient in its ability to detect the target organism(s). The deficiency of the culturing procedure is understandable when the essentially uncultivable *Treponema denticola* (28, 30) and *B. forsythus* (12, 38) are being monitored, but it would not be expected when the target organisms include the cultivable *Porphyromonas gingivalis*, *Prevotella* (*Bacteroides*) *intermedia*, and *Actinobacillus actinomycetemcomitans* species, among others.

The present research was initiated to compare the ability of several detection methods, i.e., a serial dilution anaerobic culture and/or microscopic procedure (21), a DNA probe procedure (29), and immunological reagents using both an enzyme-linked immunosorbent assay (ELISA) (22) and an indirect immunofluorescence assay (IFA) (3), to detect a selected panel of periodontopathic organisms in single subgingival plaque samples taken from periodontally diseased sites. The choice of *T. denticola*, *P. gingivalis*, *A. actinomycetemcomitans*, and *B. forsythus* was based on their reported involvement in periodontal disease (8, 16, 21, 25, 29, 30, 32), our access to probes and immunological reagents to these organisms, and the observation that three of these species, *P. gingivalis*, *T. denticola*, and *B. forsythus*, each have an enzyme(s) (17) capable of hydrolyzing benzoyl-DL-arginine-naphthylamide (BANA). Our purpose was to compare the immunological and DNA probe results with those

* Corresponding author.

obtained by the culture and/or microscopic procedure and to determine which of these procedures would best serve as the reference procedure for the detection of these and possibly other bacterial species in dental plaque. The most reliable procedure would then be used as a reference for the evaluation of an enzyme diagnostic test for some of these organisms, which is based upon the hydrolysis of BANA (17).

MATERIALS AND METHODS

Selection of patients. Patients were selected from those seeking treatment at the University of Detroit Dental Clinic at the Detroit Receiving Hospital (n = 42) and at the Periodontal Graduate Clinic at The University of Michigan School of Dentistry (n = 25). All patients were examined by a periodontist and deemed to have moderate to advanced forms of periodontal disease as judged by bone loss, by periodontal attachment loss, and by the presence of bleeding upon probing in one or more periodontal pockets. One hundred and thirty-nine samples were collected at the University of Detroit, and 65 samples were collected at the University of Michigan.

Selection of sample sites and collection of subgingival plaque. All selected pocket sites bled upon probing and were judged by the examining periodontist to be the most periodontally diseased site(s) in the patient's dentition. All selected sites were subsequently given treatment consisting of scaling, root planing, and, in many cases, Widman flap surgery. The supragingival plaque was removed from each test tooth and discarded. Then, a periodontal curette was placed at the base of the pocket, and the subgingival plaque was removed and placed in 700 μl of a reduced transport fluid (RTF), a phosphate buffer reduced with dithiothreitol (18). If there was no plaque visible in the RTF, then plaque from diseased sites on the same tooth or the adjacent proximal surface was collected and added to the RTF so as to have enough plaque material to perform each of the assays described below. The vials containing the plaque were placed in liquid nitrogen (41).

Processing of the plaque sample. The plaque samples were thawed and dispersed for 10 s with a vortex mixer. A 10-μl portion was examined by dark-field microscopy using a Zeiss microscope, and the total numbers of bacteria and total spirochetes were determined. Prior testing had shown that if the sample had fewer than 20 bacteria in 5 high-power microscopic fields (hpf), then the sample was too small to give reliable data in the following tests, and these small samples were not processed further. If the sample had ≥20 bacteria per 5 hpf, the sample was divided as follows: (i) 100 μl was added to the test buffer provided by BioTechnica Diagnostics (Cambridge, Mass.), quickly frozen, and shipped on dry ice immediately to BioTechnica for the enumeration of *P. gingivalis*, *T. denticola*, and *A. actinomycetemcomitans*; (ii) 200 μl was placed in 0.5% formaldehyde in phosphate-buffered saline (PBS) to be subsequently analyzed for *P. gingivalis*, *T. denticola*, *B. forsythus*, and *A. actinomycetemcomitans* by using both an ELISA and a fluorescent-antibody procedure; (iii) 80 μl was diluted in 8 ml of RTF, serially diluted, and cultured on selective and nonselective media for *P. gingivalis*, *B. forsythus*, and *A. actinomycetemcomitans*; (iv) 225 μl was used for a test (PerioScan; Oral-B Laboratories Inc., Redwood City, Calif.) which measures BANA hydrolysis (20).

DNA probes. Whole genomic probes (labeled with ^{32}P) to *P. gingivalis* (ATCC 33277) and *T. denticola* (ATCC 35405) and a cloned probe to *A. actinomycetemcomitans* (strain Y4 [ATCC 43718]) have been developed by BioTechnica Diagnostics (9, 35), who provide a commercial reference laboratory for the detection of these organisms in plaque samples. The probe to *A. actinomycetemcomitans* exhibited a low (i.e., 1%) hybridization to *Haemophilus aphrophilis*, *H. haemolyticus*, *H. parahaemolyticus*, and *H. influenzae* but showed no interactions with 41 other species resident in dental plaques (9, 35). The probe to *P. gingivalis* exhibited a low (i.e., 1%) hybridization to *B. forsythus*, *T. denticola*, *P. endodontalis*, and *Bacteroides oris* but did not cross-react with 41 other species resident in plaque (9, 35). Unpublished results showed that the probe for *T. denticola* did not cross-react with over 70 plaque species but did exhibit a low, (i.e., 1%) hybridization with *Wolinella recta* and *W. curva* (22a) (BioTechnica Diagnostics, technical data). BioTechnica had ascertained previously that the constituents of the RTF did not interfere with their DNA assays. The plaque samples were prepared for application to nitrocellulose filters and subsequent hybridization with the ^{32}P probes (9). The results generated by the Optimas Video Imaging System were reported as negative ($<6 \times 10^3$ cells), low ($\geq 6 \times 10^3$ but $<6 \times 10^4$ cells), moderate ($\geq 6 \times 10^4$ but $<6 \times 10^5$ cells), and high ($\geq 6 \times 10^5$ cells).

Culture and microscopic methods. A 80 μl portion of the plaque suspension was added to 8 ml of RTF and brought into the anaerobic chamber. The plaque was dispersed by 20 s of sonification using a Kontes ultrasonic disrupter (Kontes, Cincinnati, Ohio). Serial 10-fold dilutions in RTF were plated with a spiral plater (Spiral Systems Inc., Cincinnati, Ohio) on a nonselective ETSA medium (36) and on a selective vancomycin-polymyxin medium for *A. actinomycetemcomitans* (31). The ETSA plates were incubated in the chamber for 7 days at 35°C under an atmosphere of 85% N_2–10% H_2–5% CO_2, whereas the vancomycin-polymyxin plates were incubated for 7 days in a CO_2 incubator.

The plates were examined with a stereomicroscope, and the total counts, the count of black-pigmented colonies, and the count of speckled colonies were determined on ETSA plates containing between 50 and 250 to 300 colonies. Portions of the black-pigmented colonies were placed on PerioScan cards and incubated for 15 min at 55°C to measure BANA hydrolysis. Black-pigmented colonies that were BANA-positive were identified as *P. gingivalis*, as this species is the only oral black-pigmented organism known to hydrolyze BANA (17). The speckled colonies were thin filamentous rods consistent with the description of *B. forsythus* (38). Portions of these colonies were placed on the PerioScan cards, and only BANA-positive colonies were identified as *B. forsythus* (17). This identification was confirmed by demonstrating that only the BANA-positive colonies gave a positive ELISA reaction with highly specific polyclonal antisera directed against *B. forsythus* ATCC 43037. None of the *B. forsythus* isolates could be subcultured on the ETSA medium, confirming the difficulty of growing this organism (12, 38). *A. actinomycetemcomitans* was identified by its characteristic colony type on the vancomycin-polymyxin medium (31). The proportions of *P. gingivalis*, *B. forsythus*, and *A. actinomycetemcomitans* were determined by dividing their counts on the agar medium by the total counts in the ETSA plates and reported as a percentage of the viable count.

T. denticola cannot be quantitatively isolated on the ETSA medium (28). Its presence and levels in the plaque were estimated indirectly by counting spirochetes in the 10 μl of vortex-dispersed plaque sample that was used initially to screen out small samples. Twenty hpfs (100× oil immer-

sion objective lens) or 200 total bacteria were counted, depending on which event came first. Under these conditions, the volume of 1 hpf is approximately 1.9×10^{-6}/ml. The bacteria were characterized as spirochetes, fusiforms, motile rods, rods, and cocci. The proportions of each morphotype were determined by dividing the count of the morphotype by the total count, and the data were reported as a percentage of the microscopic count.

Immunological procedures. Reference strains of *A. actinomycetemcomitans* (ATCC 43718), *P. gingivalis* (ATCC 33277), and *T. denticola* (ATCC 35405) were grown as previously described (23, 40). The bacteria were harvested by centrifugation and washed three times in 0.15 M NaCl. *B. forsythus* (FDC 338 [ATCC 43037]) was grown by A. Tanner (Forsyth Dental Center, Boston, Mass.), who provided approximately 1 g (wet weight) of *B. forsythus* cells. The cells of the four species were individually resuspended in 1% formaldehyde in PBS and stored at 4°C for 4 days. The cells were then washed three times in PBS to remove formaldehyde and lyophilized.

Hyperimmune polyclonal rabbit antibodies were prepared by subcutaneous immunization of female New Zealand White rabbits with 2 mg of the lyophilized immunization antigen suspended in an aluminum hydroxide gel adjuvant at 0 and 1 week, followed by booster immunization in incomplete Freund's adjuvant at 7 weeks following the first immunization. Preimmune and immune (post-7 weeks) bleedings were obtained. The specificity of the antibodies was determined first by IFA to confirm that the antibody was staining morphologically characteristic bacterial cell types. In cases where cross-reaction was detected, the sera were absorbed against the appropriate cross-reacting isolates to produce serologically specific reagents. Briefly, heat-inactivated antisera were diluted 1:2 with PBS and then incubated with equal volumes of formalin-killed bacteria for 2 h at room temperature, followed by a 4°C incubation overnight. The samples were then centrifuged at 5,000 × *g* for 10 min, and the pelleted bacteria were discarded. Each serum sample was subjected to this absorption scheme for four total cycles. The efficacy of this absorption scheme was then reevaluated in the ELISA. The resulting antisera were monospecific for the immunizing antigen when used with working titers of 1/1,000.

Indirect immunofluorescence. After vortexing, 10 μl of plaque samples or pure cultures was applied to wells of 12-well immunofluorescence slides. Each slide contained a positive (pure culture) and a negative control. The remaining 10 wells of each slide received a sample of the plaque suspension. The slides were air dried at 37°C, and the specimens were gently heat fixed. The appropriate antibacterial antibody was diluted in PBS containing 2% bovine serum albumin (PBS–2% BSA), and 10.0 μl was applied to all wells except the fluorescein isothiocyanate conjugate control. The slides were incubated in a humid chamber for 30 min at room temperature, given two 5-min washes in PBS, and rinsed in distilled water. Goat anti-rabbit fluorescein isothiocyanate conjugate (Zymed) diluted in PBS–2% BSA (10 μl per well) was next applied, and the slides were again incubated for 30 min in a humid chamber at room temperature prior to washing and rinsing as described above. The slides were mounted with glycerol in PBS (2:1, vol/vol) containing paraphenylenediamine (pH 9.0) and sealed with a coverslip and nail polish prior to microscopy. Randomly selected hpfs were examined, and the fluorescing microorganisms were counted with a Leitz Dialux microscope equipped with a Ploempak 2.3 fluorescence illuminator for epifluorescence. Staining was considered positive for the selected microorganisms if it revealed strongly fluorescent cells with well-defined outlines and dark or lightly shining centers, constituting more than 1% of the total count (40).

ELISA methodology. The samples used for indirect immunofluorescence were utilized in a slot blot assay. Prior to the assay the samples were subjected to ultrasonification (3 to 5 s at the maximum power setting of the Kontes sonifier; Kontes Instruments) to disrupt aggregates of plaque particles. Nitrocellulose sheets (Schleicher & Schuell) were soaked for 15 min in Tris-buffered saline (TBS) prior to insertion in the slot blot manifold (Schleicher & Schuell). Standards (pure cultures) or undiluted plaque samples were applied (10 μl) to each well of the slot blot manifold and processed (40). Microbial detection and quantitation was performed after the nitrocellulose membrane was first immersed in TBS containing 0.5% nonfat dried milk (BLOTTO) for 30 to 60 min to block unoccupied binding sites on the nitrocellulose membrane. The appropriate antibacterial antibody, diluted in TBS-Tween 20 (TBS-T) containing 0.5% BLOTTO, was applied, and the mixture was allowed to incubate for 1 h. Following three 5-min washes in TBS-T, goat anti-rabbit immunoglobulin G (heavy plus light chains) conjugated to alkaline phosphatase (Bio-Rad Laboratories) diluted in TBS-T containing 0.5% BLOTTO was applied, and the mixture was incubated for 1 h. After all washes, the 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP)-nitroblue tetrazolium (NBT) substrate solution (Kirkegaard & Perry Laboratories) was applied, and color development was allowed to proceed to its maximum. A score of 0 indicated no color, a score of 1 represented a concentration of at least 2×10^4 CFU, a score of 2 represented from 2×10^4 to $<3 \times 10^5$ CFU, and a score of 3 represented at least 3×10^5 CFU (40).

Statistical analysis. The bacteriological and immunological measurements were recorded as ordinal responses ranging from no detectable levels to high levels of bacteria. For all analyses, the ordinal responses were dichotomized, with a 0 indicating the absence and a 1 indicating the presence of bacteria. The level of detection of the bacteria (presence) for the various analyses were as follows: cultivation, $\geq 2 \times 10^3$ CFU (≥0.2% of the viable flora); microscopic count, $\geq 10^4$ CFU; DNA probes, $\geq 6 \times 10^3$ CFU; IFA and ELISA, $\geq 10^4$ CFU. Empirical estimators of sensitivity, specificity, and accuracy were used in Tables 2 through 5. The sensitivity was estimated as the number of positive results in the test procedure divided by the number of positive results in the reference procedure. The specificity was estimated as the number of negative results in the test procedure divided by the negative results in the reference procedure. The accuracy was estimated by the number of true positives (sensitivity) plus true negatives (specificity) divided by the total number of results.

A site was classified as a true positive when three or more of the four different diagnostics tested positive and as a true negative when three or more of the four different diagnostics tested negative. Since no DNA probes were available for *Bacteroides forsythus*, a true positive was defined as two or three diagnostics testing positive for any given plaque sample. The sensitivity, specificity, and accuracies of the various detection methods and their respective standard errors were estimated by using jackknifing methodology (11, 15). The statistical significance of differences in sensitivity, specificity, or accuracies was investigated by using the *z* statistic (*z* = difference between the estimates divided by the square

TABLE 1. Presence or absence of selected periodontopathic organisms in subgingival plaques taken from diseased sites

Detection methodology	% of sites[a]									
	P. gingivalis		*B. forsythus*		*T. denticola*		Spirochetes		*A. actinomycetemcomitans*	
	−	+	−	+	−	+	−	+	−	+
Culture	56.7	43.3	74.6	25.4					86.4	13.6
Microscopic							1	99		
DNA	17.4	82.6			13.4	86.6			85.1	14.9
ELISA	11.4	88.6	35.9	64.1	17.9	82.1			51.0	49.0
IFA	36.0	64.0	6.1	93.9	42.1	57.9			44.9	55.1

[a] Percentage of sites in which the indicated species was absent (−) or present (+) in the plaque. Values in boxes show comparable results obtained by two separate detection methodologies.

root of the sum of the variances). A Bonferroni adjustment was performed to obtain an overall error rate of 0.05.

RESULTS

A total of 204 plaque samples were collected from 67 patients. All sampled sites bled upon probing, had an average probing depth of 6.4 ± 1.8 mm, and were considered by the clinician to be the most diseased site in either the tooth quadrant or the dentition and to be in need of treatment. The prevalence of the four monitored species in these plaques varied as a function of both the species and the detection method (Table 1). *P. gingivalis* and *T. denticola* were the most prevalent species, being present in 82 to 89% of the plaques (DNA probe and ELISA reactions). *A. actinomycetemcomitans* was the least prevalent species by all detection methods and was present in only 14% of the plaques as judged by the DNA probes and cultural results. When *A. actinomycetemcomitans* was detected, the plaque was always colonized by either *P. gingivalis*, *T. denticola*, or *B. forsythus* alone or in combination.

The lowest prevalence values were observed with the cultural analysis, whereas the highest values were obtained with one of the immunological procedures. The immunological procedures, although they used the same antibodies, gave comparable prevalence values only in the case of *A. actinomycetemcomitans*. The DNA probes and ELISA reactions gave comparable prevalence rates for *P. gingivalis* and *T. denticola*, and the cultural and DNA probes were in agreement concerning the prevalence of *A. actinomycetemcomitans* (Table 1). None of the detection methods were in agreement concerning the prevalence of *B. forsythus*. The IFA indicated that *B. forsythus* was present in 94% of the plaques, a value which contrasted sharply with the 25% prevalence seen with the cultural approach (Table 1).

These findings indicated that the prevalence of an organism in a plaque site was a function of the detection method and the organisms themselves and that none of these methods could be considered to be a primary standard. In fact, the cultural method, which is generally used as the gold or primary standard for the other techniques, consistently yielded the lowest prevalence values for the three cultivable species. To determine the degree of agreement among the various procedures, all the detection methods were sequentially analyzed from the perspective of being both a reference procedure and the test procedure for each of the monitored organisms (Tables 2 to 5).

If the cultural procedure was set as the reference procedure and *P. gingivalis* was the target organism, when the cultures were positive for *P. gingivalis* the DNA probe and the ELISA test for *P. gingivalis* were also positive in about 90% of the plaques, i.e., a high sensitivity (Table 2). However, the specificity was about 20%, indicating that 80% of the plaques that were culture negative had *P. gingivalis* as detected by both the DNA probe and ELISA antibody, i.e., "false positive." This resulted in an accuracy of about 50%, indicating that if the cultural data were the primary standard, then the information obtained with the DNA probe and either ELISA or IFA would only be correct in half of the plaque samples.

When the DNA probe results were taken as the reference standard for *P. gingivalis*, the culturing procedure detected *P. gingivalis* in 47% of the plaques in which the DNA probe showed it to be actually present. If the antibodies to *P. gingivalis*, measured by either ELISA or IFA, were used as the reference test, the culturing procedure showed a sensi-

TABLE 2. Comparisons of various microbiological measuring procedures in their ability to detect *P. gingivalis* in subgingival plaques

Test procedure	Result[a] for reference procedure											
	Culture			DNA probes			ELISA			IFA		
	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity
Culture				51.7	47.0 (78/166)	74.3 (26/35)	48.0	44.9 (80/178)	70.8 (17/24)	51.2	45.3 (58/128)	61.6 (45/73)
DNA probes	51.7	89.7 (78/87)[b]	22.8 (26/119)				82.1[b]	86.5 (154/178)[b]	47.8 (11/23)	70.5	91.4 (117/128)[b]	33.3 (12/36)
ELISA	48.0	92.0 (80/87)[b]	14.8 (17/115)	82.1[b]	92.8 (154/166)[b]	31.4 (11/35)				66.7	93.0 (119/128)[b]	20.6 (15/73)
IFA	51.2	67.4 (58/86)	39.1 (95.115)	70.5	70.9 (117/165)	68.6 (24/35)	66.7	67.2 (119/177)	62.5 (15/24)			

[a] Sensitivity (true positive) = (no. positive in test procedure/no. positive in reference procedure) × 100. Specificity (true negative) = (no. negative in test procedure/no. negative in reference procedure) × 100. Accuracy = [(sensitivity + specificity)/total no. of plaques in sample] × 100.

[b] Value might have utility as a diagnostic test.

TABLE 3. Comparisons of various microbiological measuring procedures in their ability to detect *T. denticola* in subgingival plaques

Test procedure	Result[a] for reference procedure											
	Microscopic analysis			DNA probes			ELISA			IFA		
	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity
Microscopic analysis				85.6[b]	98.9 (172/174)[b]	0 (0/27)	81.2[b]	98.8 (164/166)[b]	0 (0/36)	57.1	98.3 (113/115)[b]	0 (0/83)
DNA probes	85.6[b]	86.4 (172/199)[b]	0 (0/2)				72.6	86.1 (142/165)[b]	11.1 (4/36)	51.8	83.3 (95/114)[b]	8.4 (7/83)
ELISA	81.2[b]	82.0 (164/200)[b]	0 (0/2)	72.6	81.6 (142/174)[b]	14.8 (4/27)				70.2	94.8 (109/115)[b]	36.1 (30/83)
IFA	57.1	57.7 (113/196)	0 (0/2)	51.8	55.6 (95/171)	26.9 (7/26)	70.2	67.3 (109/162)	83.3 (30/36)[b]			

[a] See Table 2, footnote *a*.
[b] Value might have utility as a diagnostic test.

tivity of only 45% (Table 2). We next compared all the detection methods relative to each other for their ability to detect *P. gingivalis* in these plaque samples. When the DNA probe results served as the reference standard, the best agreement was with the ELISA results, where a 93% sensitivity and an 82% accuracy were observed (Table 2). When the ELISA results were the reference standard, the best agreement was with the DNA probes, where a sensitivity of 87% and an accuracy of 82% were observed. When the IFA results were the reference standard, the sensitivity with the DNA probes and ELISA was 91 to 93%, but a low specificity reduced the accuracy values to 67 to 70% (Table 2).

A similar analysis was performed for *T. denticola*. In this case, the culturing results were replaced by the microscopic count for spirochetes, a situation which would inflate the detection values, as not all spirochetes are *T. denticola*. The microscopic count showed good agreement with both the ELISA and DNA results, with sensitivities of about 82 to 86% and accuracies of 81 to 86%, but the agreement with the IFA results was low, i.e., 57% accuracy (Table 3). The specificity was 0 because none of the 204 plaques examined was negative for both spirochetes and *T. denticola*. The accuracies between the DNA probes and immunological tests for *T. denticola* were from 50 to 70%, regardless of which procedure served as the reference standard. These lower accuracies were a function of poor specificity due to a high number of false positives (Table 3). Results for the two immunological assays differed despite the fact that the same antibody was used in both procedures.

When the cultural procedure was the reference standard for *B. forsythus*, the immunological tests were accurate in only 29 to 38% of the comparisons (Table 4). When either one of the immunological assays served as the reference standard, the accuracy improved to 60%. Almost all plaques that were positive for *B. forsythus* by either the cultural or the ELISA procedure were also positive for *B. forsythus* by IFA. However, when IFA served as the reference standard, only 25% of the plaques that were IFA positive yielded cultivable *B. forsythus*, and 63% had *B. forsythus* that could be detected by ELISA.

When the cultural procedure was the reference standard for *A. actinomycetemcomitans*, there was an accuracy of 75% when compared with the DNA probe results, but only an accuracy of 43 to 48% when compared with the ELISA and IFA results (Table 5). The relatively high accuracy in the comparison with the DNA probe was due to a specificity of 85%, as the sensitivity was only 14%. The same antibody to *A. actinomycetemcomitans*, compared with either the ELISA or the IFA procedure as the reference, had an accuracy value of 67% (Table 5).

These one-on-one comparisons indicated that the cultural approach gave lower detection frequencies for *P. gingivalis*, *B. forsythus*, and *A. actinomycetemcomitans* than did the other detection methods. This raised the question as to which results, those obtained by culture or those obtained by the DNA probes and immunological reagents, were the most credible. This issue was addressed by looking at the prevalence profile of the target organism in the plaque, as determined by all the detection methods. It was assumed that if a species was not detected in the plaques by three or four methods, then this species was most likely not present in the plaques. Likewise, if the species was detected in the plaque by three or four methods, then it was most likely present. This prevalence profile for the targeted organisms is shown in Table 6. *P. gingivalis* and *T. denticola* or spirochetes were detected by three or four of the detection methods in 68 to 81% of the plaque samples, whereas *A. actinomycetemcomitans* was detected in only 14% of the plaque samples (Table 6). (*B. forsythus* was not included in this analysis because of the absence of DNA probe data.) Only 1% of the plaques did not have spirochetes or *T. denticola* and 13% did not have *P. gingivalis*, but 54% did not have *A. actinomycetemcomitans*.

The various methods were then compared for sensitivity, specificity, and accuracy with a true positive defined as

TABLE 4. Comparisons of various microbiological measuring procedures in their ability to detect *B. forsythus* in subgingival plaques

Test procedure	Result[a] for reference procedure								
	Culture			ELISA			IFA		
	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity
Culture				37.7	21.3 (27/127)	66.7 (48/72)	29.1	25.7 (48/187)	83.3 (10/12)[b]
ELISA	37.7	52.9 (27/51)	32.4 (48/148)				59.7	62.7 (116/185)	7.1 (1/11)
IFA	29.1	96.0 (48/50)[b]	6.7 (10/149)	59.7	92.1 (116/126)[b]	1.4 (1/69)			

[a] See Table 2, footnote *a*.
[b] Value might have utility as a diagnostic test.

TABLE 5. Comparisons of various microbiological measuring procedures in their ability to detect *A. actinomycetemcomitans* in subgingival plaques

Test procedure	Result[a] for reference procedure											
	Culture			DNA probes			ELISA			IFA		
	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity
Culture				75.1	13.3 (4/30)	86.0 (147/171)[b]	48.0	11.3 (11/97)	83.2 (84/101)[b]	43.4	11.1 (12/108)	83.0 (73/88)[b]
DNA probes	75.1	14.3 (4/28)	85.0 (147/173)				56.6	20.6 (20/97)	91.1 (92/101)[b]	52.8	21.3 (23/108)	92.0 (80/87)[b]
ELISA	48.0	32.3 (11/28)	49.4 (84/170)	56.6	69.0 (20/29)	54.5 (92/169)				66.7	63.9 (69/108)	70.2 (59/84)
IFA	43.4	44.4 (12/27)	43.2 (73/169)	52.8	76.7 (23/130)	48.5 (80/165)	66.7	73.4 (69/94)	60.2 (59/98)			

[a] See Table 2, footnote *a*.
[b] Value might have utility as a diagnostic test.

being detected by four or three of the methods and a true-negative defined as being detected by zero or one of the methods. This analysis showed that *P. gingivalis* could be detected in the plaque samples with an accuracy of 90 to 96% when the DNA probe and antibodies were used (Table 7). All three accuracy values obtained with these reagents were significantly higher than the 61% accuracy value observed with the cultural method. This low accuracy value observed with the cultural method was due to a low sensitivity (i.e., 55%), indicating that the other methodologies were often positive when *P. gingivalis* could not be cultured.

A. actinomycetemcomitans could be detected in the plaques with an accuracy of 94% when the DNA probe was used and with an accuracy of 88% when the antibody was evaluated in the ELISA procedure. The accuracy obtained with the DNA probe was significantly higher than the 76 to 79% accuracies observed with the IFA procedure and the cultural method (Table 7). The cultural method was conspicuous in having a significantly lower sensitivity than the other methods. The microscopic count detected spirochetes with an accuracy of 97%, which was identical to the 97% accuracy of *T. denticola* detection with a polyclonal antibody in the ELISA procedure (Table 7). However, the same antibody when used in the IFA procedure was significantly less accurate in detecting *T. denticola*.

DISCUSSION

The results of the present investigation indicate that there are no gold standards for the detection of *P. gingivalis*, *T. denticola*, *B. forsythus*, or *A. actinomycetemcomitans* in subgingival plaque samples. In fact, the traditional standard used in periodontal microbiology, the serial dilution anaerobic culturing procedure (13, 24, 33), appeared to be the poorest detection method, if we accept the higher detection frequencies with both the DNA probes and polyclonal antibodies as more indicative of reality (Tables 1 and 6). In the absence of a reference standard, there is the equal possibility that each detection procedure reflects reality. We attempted to overcome this problem by defining reality as the consensus that was found when several independent approaches looked at the same event. In this case, we considered a true colonization more likely if all or the majority of the tests indicated that the organism(s) was present. Because this type of analysis involved the elimination of plaques where two tests gave a positive finding and two tests gave a negative finding, we had introduced a selection bias which would result in biased estimates of sensitivity, specificity, and accuracy. This was considered an acceptable strategy, but it illustrates how problematic the estimation of the characteristics of a diagnostic test is in the absence of a primary reference standard (27, 39) (which is the most common event, as the presence of such a standard is the exception) (1, 14).

We could perform this consensus analysis for *P. gingivalis* and *A. actinomycetemcomitans*, as we had three (four, if we count ELISA and IFA as separate entities) distinct approaches for detecting these organisms. The analyses for *T. denticola* and *B. forsythus* were incomplete in this regard, as

TABLE 6. Prevalence of periodontopathic bacteria in subgingival plaques as determined by four detection methods

Prevalence profile[a]	*P. gingivalis* (n = 192)[b]		*T. denticola* or spirochetes (n = 191)[c]		*A. actinomycetemcomitans* (n = 186)[d]		*B. forsythus* (n = 164)
	%	No. of plaques	%	No. of plaques	%	No. of plaques	No. of plaques
Negative	13		1		54		
−−−− or −−−		8		0		46	0
−−−+ or −−+		16		2		55	48
−−++		38		35		60	
Positive	68		81		14		
−+++ or −++		79		64		24	25
++++ or +++		51		90		1	91

[a] True negative is defined as a combination of (−−−−) and (−−−+). True positive is defined as a combination of (−+++) and (++++). Each symbol represents a detection method. Series of three symbols (e.g., −−−) apply to *B. forsythus* only.
[b] Accuracy = 80.2%.
[c] Accuracy = 81.7%.
[d] Accuracy = 67.8%.

TABLE 7. Comparisons of microbiological procedures in their ability to detect selected periodontopathic species in subgingival plaques[a]

Detection method	*P. gingivalis*			*T. denticola* or spirochetes			*A. actinomycetemcomitans*		
	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy	Sensitivity	Specificity	Accuracy
Culture/microscopic[b]	54.6 (6.5)	95.8 (4.2)	61.0 (5.8)	98.7 (1.3)	Not calculable	97.4 (1.5)	35.9 (11.7)	90.0 (3.5)	79.4 (4.4)
DNA probes	97.7 (1.7)	88.1 (7.3)	96.1 (1.8)	88.3 (3.3)	Not calculable	88.4 (3.3)	72.2 (10.4)	99.0 (1.0)	93.6 (2.4)
ELISA	97.7 (1.7)	54.2 (13.3)	90.9 (3.3)	97.4 (1.2)	Not calculable	97.5 (1.2)	96.0 (4.0)	86.2 (4.6)	88.1 (4.0)
IFA	89.2 (3.8)	96.0 (4.2)	90.3 (3.3)	74.1 (5.7)	Not calculable	74.4 (5.5)	100.0 (0)	70.4 (4.9)	76.2 (4.0)

[a] Value in parentheses is standard error. Other values are percents. Values connected by brackets are significantly different ($P < 0.05$). Experimentwise error rate = 0.05.

[b] Culture used for *P. gingivalis* and *A. actinomycetemcomitans*; microscopic count used for *T. denticola* and spirochetes.

the microscopic examination will only detect spirochetes and there was no DNA probe for *B. forsythus*. This approach showed 27% of the plaques to be positive for *P. gingivalis* by all four assays, whereas only 0.5% were positive for *A. actinomycetemcomitans*. Four percent of the plaques did not have *P. gingivalis*, and 25% did not have *A. actinomycetemcomitans* (Table 6). We relaxed the definitions of true positive and true negative so as to include as positive a positive result in three of the four methods and as negative a negative result in three of the four methods. We also included the microscopic data for spirochetes in the analysis. This approach showed the DNA probes and immunological reagents to be superior, in some cases significantly superior ($P < 0.05$), to the cultural approach for the detection of *P. gingivalis* and *A. actinomycetemcomitans* and to be comparable to the microscopic approach in the detection of *T. denticola* (Table 7).

The superiority of the DNA probes and antibodies relative to the culturing results could reflect the possibility that the higher detection frequencies with the DNA probes and antibodies were artifactual, due to the lack of specificity (in the microbiological sense) of these reagents. The DNA probes for *P. gingivalis* and *A. actinomycetemcomitans* did not show cross-reactivity with over 40 bacterial species that might be expected to be found in plaque (9, 35). The DNA probe to *T. denticola* exhibited a low (i.e., 1%) hybridization with *W. recta* and *W. curva*, of over 68 species tested (22a). These findings suggest that the DNA probes did not detect species other than the sought-after organisms. The antibodies used for the ELISA and IFA exhibited no cross-reactions with related species, except that the *T. denticola* antiserum showed weak cross-reactions in the IFA with *Treponema vincentii*, *Treponema socranskii*, and a treponemal strain which could not be identified to the species level (3, 40). These weak cross-reactions could be visually differentiated from the strong IFA reactions seen with *T. denticola*, and because the detection frequency of *T. denticola* by IFA was lower than that observed with the DNA probe and ELISA (Table 2), they were not thought to be a confounding factor. The superior performance of the antibodies to *T. denticola* and *P. gingivalis* in the ELISA procedure relative to the IFA procedure (Tables 1 and 7) may be attributable to the way the plaque was dispersed (40). In the IFA procedure, the plaque is dispersed by using relatively gentle mechanical means so as to maximize cell integrity. This could leave clumps of organisms in which the antigens would not be accessible to the antibody. In the ELISA procedure, the plaques are lysed by sonification, permitting the formation of a uniform suspension in which the antigens would have greater access to the antibodies.

The inability of the cultural procedures to detect certain plaque species has been noted previously (12, 28). Spirochetes can account for 40% of the microscopic count but are not grown by cultural procedures (16, 25, 28, 30). *B. forsythus* was found in seven of seven plaques when analyzed with monoclonal antibodies by an IFA but could not be detected by cultural methods (12). Zambon et al. (42) found that plaque cultures were positive for *P. gingivalis* in 26 to 66% of the plaques that were positive for *P. gingivalis* by IFA. Savitt et al. (29) found that the cultural method underestimated the prevalence of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* in plaque samples relative to their respective DNA probes.

The cultural procedure has many methodological problems when used in periodontal microbiology. The serial-dilution anaerobic culturing procedure will recover about 20 to 70% of the microscopic count obtained on the same plaque sample (13, 24, 33). The inability of the cultural count to coincide with the microscopic count is usually attributed to the presence of uncultivable organisms, such as the various spirochetal species, which can average about 50% of the flora removed from diseased sites (16), and to the presence of dead organisms. However, there are additional method errors, with unknown error rates, that can be associated with the sampling procedure (5, 24, 25, 37), the media used (25, 34), the degree of anaerobiosis employed (13), and the type of dispersal procedures used (28). The magnitude of these errors may vary with each of the cultivable species found in the plaque (24) and could be as high as fivefold with some of the more fastidious species (25).

In contrast to the other detection methods, a positive finding based on cultivation can be confirmed by subsequent testing. The *P. gingivalis* and *B. forsythus* isolates were confirmed by the BANA test and, in the case of *B. forsythus*, by our antibody reagent. *A. actinomycetemcomitans* was identified only by colonial morphology, which raises the possibility that our counts of this species were not as reliable as those of the BANA-positive species. This possibility plus the fact that a selective medium was used, which could have reduced the actual number of *A. actinomycetemcomitans* that were isolated, could explain the low sensitivities observed for this organism when culturing was compared with the other detection methods (Table 5).

In the present study, as the plaque samples were frozen in liquid nitrogen prior to culturing, it is possible that this procedure may have adversely affected the viable count. The plaques were frozen in liquid nitrogen because of the convenience this provided for the collection and processing of the samples and because prior reports had indicated that gram-negative anaerobic organisms of the type found in dental plaque could quantitatively survive this procedure (6, 10, 40). An unexpected benefit of the usage of liquid nitrogen related to the preservation of antigens on the bacterial surfaces. Previously we have had to add protease inhibitors to the

plaque in order to prevent endogenous proteases from degrading surface antigens reactive with our antibodies (21a), but this was not necessary with the frozen plaque samples.

If we accept that when three or more divergent reference methods agree in their detection of a plaque species as the indication of reality, then it would appear that DNA probes and/or ELISA antibodies can reliably detect *P. gingivalis*, *A. actinomycetemcomitans*, *T. denticola*, and *B. forsythus* (Tables 6 and 7). Improvements in the immunological reagents and probes, possibly including the mating of fluorescein-labelled antibodies and probes with a sensitive detection technique such as the flow cytometer (26), could result in these procedures becoming the reference standards for future microbiological diagnostic tests.

ACKNOWLEDGMENTS

This research was supported by U.S. Public Health Service Grant DE06030 from the National Institute of Dental Research and by a grant from Oral-B Laboratories Inc., Redwood City, Calif.

We thank Carol Gerlach for the preparation of the manuscript.

REFERENCES

1. **Begg, C. B.** 1987. Biases in the assessment of diagnostics tests. Stat. Med. **6**:411.
2. **Bonita, Y., J. J. Zambon, R. J. Genco, and M. E. Neiders.** 1985. Rapid identification of periodontal pathogens in subgingival plaque: comparison of indirect immunofluorescence microscopy with bacterial culture for detection of *Actinobacillus actinomycetemcomitans*. J. Dent. Res. **64**:793–798.
3. **Bretz, W. A., D. E. Lopatin, and W. J. Loesche.** 1990. Benzoyl-arginine naphthylamide (BANA) hydrolysis by *Treponema denticola* and/or *Bacteroides gingivalis* in periodontal plaques. Oral Microbiol. Immunol. **5**:275–279.
4. **Cochrane, A. L., and W. W. Holland.** 1971. Validation of screening procedures. Br. Med. Bull. **27**:3–8.
5. **Dahlen, G., S. Renvert, M. Wickström, and J. Egelberg.** 1990. Reproducibility of microbiological samples from periodontal pockets. J. Clin. Periodontol. **17**:73–77.
6. **Dan, M., J. Richardson, M. D. Miliotis, and H. J. Koomhof.** 1989. Comparison of preservation media and freezing conditions for storage of faeces. J. Med. Microbiol. **28**:151–154.
7. **Dix, K., S. M. Watanabe, S. McArdle, D. I. Lee, C. Randolph, B. Moncla, and D. E. Schwartz.** 1990. Species-specific oligodeoxynucleotide probes for the identification of periodontal bacteria. J. Clin. Microbiol. **28**:319–323.
8. **Dzink, J. L., S. S. Socransky, and A. D. Haffajee.** 1988. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J. Clin. Periodontol. **15**: 316–323.
9. **French, C. K., E. D. Savitt, S. L. Simon, S. M. Eklund, M. C. Chen, L. C. Klotz, and K. K. Vaccaro.** 1986. DNA probe detection of periodontal pathogens. Oral Microbiol. Immunol. **1**:58–62.
10. **Gilmour, M. N., G. Turner, R. G. Berman, and A. K. Krenzer.** 1978. Compact liquid nitrogen storage system yielding high recoveries of gram-negative anaerobes. Appl. Environ. Microbiol. **35**:84–88.
11. **Gladen, B.** 1979. The use of jackknife to estimate proportions from toxicological data in the presence of litter effects. J. Am. Stat. Assoc. **74**:278–282.
12. **Gmür, R.** 1988. Applicability of monoclonal antibodies to quantitatively monitor subgingival plaques for specific bacteria. Oral Microbiol. Immunol. **3**:187–191.
13. **Gordon, D. F., M. Stutman, and W. J. Loesche.** 1971. Improved isolation of anaerobic bacteria from the gingival crevice area of man. Appl. Microbiol. **21**:1046–1050.
14. **Hui, S. L., and S. D. Walter.** 1980. Estimating the error rates of diagnostic tests. Biometrics **6**:167.
15. **Hujoel, P. P., W. J. Loesche, and T. A. DeRouen.** 1990. Assessment of relationships between site-specific variables. J. Periodontol. **61**:368–372.
16. **Loesche, W. J.** 1988. The role of spirochetes in periodontal disease. Portside Symp. Adv. Dent. Res. **2**:275–283.
17. **Loesche, W. J., W. A. Bretz, D. Kerschensteiner, J. Stoll, S. S. Socransky, P. Hujoel, and D. E. Lopatin.** 1990. Development of a diagnostic test for anaerobic periodontal infections based on plaque hydrolysis of benzoyl-DL-arginine-naphthylamide. J. Clin. Microbiol. **28**:1551–1559.
18. **Loesche, W. J., R. N. Hockett, and S. A. Syed.** 1972. The predominant cultivable flora of tooth surface plaque removed from institutionalized subjects. Arch. Oral Biol. **17**:1311–1326.
19. **Loesche, W. J., and P. P. Hujoel.** 1991. Microbiological based diagnostic tests for periodontitis. Considerations in regard to sensitivity, specificity and accuracy, p. 417–440. *In* N. Johnson (ed.), Periodontal diseases: markers of disease susceptibility and activity. Cambridge University Press, Cambridge.
20. **Loesche, W. J., D. E. Lopatin, J. Giordano, G. Alcoforado, and P. P. Hujoel.** 1991. Comparison of the benzoyl-DL-arginine-naphthylamide (BANA) test, DNA probes, and immunological reagents for ability to detect anaerobic periodontal infections due to *Porphyromonas gingivalis*, *Treponema denticola*, and *Bacteroides forsythus*. J. Clin. Microbiol. **30**:427–433.
21. **Loesche, W. J., S. A. Syed, E. Schmidt, and E. C. Morrison.** 1985. Bacterial profiles of subgingival plaques in periodontitis. J. Periodontol. **56**:447–456.
21a. **Lopatin, D. E.** Unpublished data.
22. **Lopatin, D. E., F. N. Smith, S. A. Syed, and E. C. Morrison.** 1983. The effect of periodontal therapy on lymphocyte blastogenesis to plaque associated microorganisms. J. Periodontal Res. **18**:93–102.
22a. **Maher, T. E.** Personal communication.
23. **Mangan, D. F., B. E. Laughon, B. Bower, and D. E. Lopatin.** 1982. In vitro lymphocyte blastogenic responses and titers of humoral antibodies from periodontitis patients to oral spirochete isolates. Infect. Immun. **37**:445–451.
24. **Mombelli, A., C. E. Minder, F. A. Gusberti, and N. P. Lang.** 1989. Reproducibility of microscopic and cultural data in repeated subgingival plaque samples. J. Clin. Periodontol. **16**:434–442.
25. **Moore, W. E. C.** 1987. Microbiology of periodontal disease. J. Periodontal Res. **22**:335–341.
26. **Obernesser, M. S., S. S. Socransky, and P. Stashenko.** 1990. Limit of resolution of flow cytometry for the detection of selected species. J. Dent. Res. **69**:1592–1598.
27. **Payne, W. J., D. L. Marshall, R. Y. Shockley, and W. J. Martin.** 1988. Clinical laboratory applications of monoclonal antibodies. Clin. Microbiol. Rev. **1**:313–319.
28. **Salvador, S. L., S. A. Syed, and W. J. Loesche.** 1987. Comparison of three dispersion procedures for quantitative recovery of cultivable species of subgingival spirochetes. J. Clin. Microbiol. **25**:2230–2232.
29. **Savitt, E. D., M. N. Strzempko, K. K. Vaccaro, W. J. Peros, and C. K. French.** 1988. Comparison of cultural methods and DNA probe analysis for the detection of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in subgingival plaque samples. J. Periodontol. **59**:431–438.
30. **Simonson, L. G., C. H. Goodman, J. J. Bial, and H. E. Morton.** 1988. Quantitative relationship of *Treponema denticola* to severity of periodontal disease. Infect. Immun. **56**:726–728.
31. **Slots, J.** 1986. Rapid identification of important periodontal microorganisms by cultivation. Oral Microbiol. Immunol. **1**:48–57.
32. **Slots, J., C. Hapstrom, B. Rosling, and G. Dahlen.** 1985. Detection of *Actinobacillus actinomycetemcomitans* and *Bacteroides gingivalis* in subgingival smears by the indirect fluorescent-antibody technique. J. Periodontal Res. **20**:613–620.
33. **Socransky, S. S., R. J. Gibbon, A. C. Dale, L. Burtnick, E. Rosenthal, and J. B. MacDonald.** 1963. The microbiota of the gingival crevice area of man. I. Total microscopic and viable counts and counts of specific organisms. Arch. Oral Biol. **8**:275–280.
34. **Socransky, S. S., A. D. Haffajee, G. L. F. Smith, and J. L. Dzink.** 1987. Difficulties encountered in the search for the etiologic agents of destructive periodontal diseases. J. Clin. Periodontol. **14**:588–593.

35. **Strzempko, M. N., S. L. Simon, C. K. French, J. A. Lippke, F. F. Raia, E. D. Savitt, and K. K. Vaccaro.** 1987. A cross-reactivity study of whole genomic DNA probes for *Haemophilus actinomycetemcomitans*, *Bacteroides intermedius* and *Bacteroides gingivalis*. J. Dent. Res. **66**:1543–1546.
36. **Syed, S. A., M. Svanberg, and G. Svanberg.** 1980. The predominant cultivable flora of gingivitis associated plaque of beagle dogs. J. Periodontal Res. **15**:123–131.
37. **Tanner, A. C., and J. M. Goodson.** 1986. Sampling of microorganisms associated with periodontal disease. Oral Microbiol. Immunol. **1**:15–22.
38. **Tanner, A. C. R., M. N. Strzempko, C. A. Belsky, and G. A. McKinley.** 1985. API ZYM and API-An-Ident reactions of fastidious oral gram-negative species. J. Clin. Microbiol. **22**: 333–335.
39. **Tenover, F. C.** 1988. Diagnostic deoxyribonucleic acid probes for infectious disease. Clin. Microbiol. Rev. **1**:82–101.
40. **van Poperin, N., and D. E. Lopatin.** 1991. Slot immunoblot assay for detection and quantification of periodontal disease-associated microorganisms in dental plaque. J. Clin. Microbiol. **29**: 2554–2558.
41. **Wilson, R. F., A. Woods, and F. P. Ashley.** 1984. The effect of storage in liquid nitrogen on the recovery of human dental plaque bacteria. Arch. Oral Biol. **29**:941–944.
42. **Zambon, J. J., H. S. Reynolds, P. Chen, and R. J. Genco.** 1985. Rapid identification of periodontal pathogens in subgingival dental plaque. Comparison of indirect immunofluorescence microscopy with bacterial culture for detection of *Bacteroides gingivalis*. J. Periodontol. **56**(Suppl.):32–40.